# AGENT AND PROCESS FOR ISOLATION OF EXTRA-CHROMOSOMAL NUCLEIC ACIDS

## Field of the Invention

The invention relates to compositions and methods for isolating extra-chromosomal nucleic acids from biological materials. More particularly, the invention relates to plasmid DNA isolation methods employing non-toxic chaotropic agents.

## Background of the Invention

Propagation and isolation of extra-chromosomal DNA is a crucial process in genetic cloning and engineering. Extra-chromosomal DNA is distinct from chromosomal or genomic DNA that forms the chromosomes. In eukaryotic and prokaryotic cells, genomic DNA is attached or bound to a membrane. In eukaryotes, such as mammalian or yeast cells, the genomic DNA is contained within and bound to the nuclear membrane. In prokaryotes, such as bacteria, genomic DNA is bound to the plasma membrane surrounding the cytoplasm. While all cells contain genomic DNA, only some have extra-chromosomal DNA. As the name implies, extra-chromosomal DNA is not attached to the chromosomal (genomic) DNA, and as such, is not attached to cell membranes. Extra-chromosomal DNA may be naturally occurring or artificially introduced into eukaryotic or prokaryotic cells. Extra-

chromosomal DNA forms known and used in genetic engineering or cloning include, but are not limited to, plasmid, cosmid, bacterial artificial chromosome (BAC), and yeast artificial chromosome (YAC) DNA.

In order to distinguish the methods of the present invention from the prior art, it is helpful to understand the general process involved in isolation of DNA. Of particular importance is an appreciation for the differences between two uses of the term "lysis": one for the isolation of genomic DNA, the other for extra-chromosomal DNA. In the first case, cells or bacteria are lysed to harvest genomic DNA by completely disrupting all cell membranes. These membranes include the plasma membranes, which form the outer wall of the cell, and the intracellular membranes, including the nuclear membrane. In this manner, genomic DNA is released from its membrane-bound state into the lysate. In the second case, cells or bacteria are treated with an alkaline lysis, which is a more gentle and incomplete disruption of the cell membranes. With an alkaline lysis, genomic DNA remains tethered to the membranes, while extra-chromosomal DNA is released into the lysate. Sedimentation of the cellular debris into a pellet effectively removes genomic DNA from the lysate, since it remains bound to the cellular debris. In each case, RNA and a multitude of protein contaminants are also released into the lysates and must be removed. A variety of purification steps must be performed on the crude lysates to obtain isolates of the DNA of interest.

There are many well-known and widely-used methods for isolation of DNA from biological material. Biological materials can include homogenized tissue samples, yeast or bacterial cultures, tissue culture cells originating from complex

organisms such as mammals or insects but subsequently grown *in vitro*, and agarose gel containing semi-pure DNA preparations.

Methods in the prior art of extra-chromosomal DNA isolation rely upon the use of toxic or caustic agents for organic extraction of impurities, or employ high affinity columns, resins, or beads to bind extra-chromosomal DNA contained within the partially processed crude biological material prior to the washing and elution or recovery steps. Examples of conventional methods can be found in *Molecular Cloning, A Laboratory Manual* Second Edition. Sambrook, J.; Fritsch, E.F.; Maniatis, T. Cold Spring Harbor Laboratory Press, 1989, or *Current Protocols in Molecular Biology, Volume 1*, John Wiley & Sons, Inc., 1995. Conventional methods require the use of toxic chemicals, such as phenol:chloroform mixtures. Aside from safety issues, the toxic chemicals can affect quality of the DNA isolated, i.e. the DNA macromolecules may be damaged or degraded. Another disadvantage with conventional methods is that they are generally more time-consuming than those using a DNA-binding matrix. Conventional methods can take up to several days and typically require the use of expensive ultracentrifugation equipment. Commercially available kits using a binding matrix take only a few hours, but are more expensive and typically result in lower yields of DNA than with conventional methods. Examples of methods using DNA-binding materials include the Qiagen MAXI-PREP and Promega WIZARD PUREFECTION PREP kits. With either conventional methods or use of DNA-binding matrices, purity of the isolate is frequently less than optimal. Typically, the cleanest (and therefore, most useful) DNA is isolated at the expense of the quantity of DNA that can be recovered.

The present invention discloses materials and methods used in an unexpected sequence to produce high yields of optimally pure extra-chromosomal DNA. The need for toxic chemicals or DNA-binding materials is eliminated. The process can be completed in about 2-3 hours using equipment commonly available in any molecular biology laboratory, using a chaotropic agent specifically formulated for the purification of extra-chromosomal DNA.

Chaotropic agents similar to the ones used in the present invention have been previously used only in the isolation of genomic DNA. Homogenized tissue samples or cell suspensions are treated with chaotropic agents, such as those disclosed in the published application W097/05248, to lyse the cell membranes and release genomic DNA into the lysate. If such materials are used to harvest extra-chromosomal DNA, the resultant release of genomic DNA contaminates the extra-chromosomal DNA. In fact, others have attempted to use such materials for isolating plasmid DNA, but have failed to devise the needed modifications to the materials and methods. Therefore, there are no prior art references teaching the use of chaotropic agents for plasmid or other extra-chromosomal DNA preparations.

In the present invention, a chaotropic solution has been modified specifically for the isolation of extra-chromosomal DNA. However, the process of the invention does not include lysis of cell homogenates or bacterial suspensions with chaotropic agents. The cells or bacteria are lysed using a standard alkaline lysis method. The lysate is separated from the cellular debris and genomic DNA, and then treated with the chaotropic agents as a purification step. The yield of extra-chromosomal DNA isolated using the present invention materials and method is significantly greater and purer than those using conventional methods. The time of isolation using the present

invention method is comparable to those dependent upon the use of DNA binding matrices. The yield is also enhanced because the method of the present invention is not influenced by the electrostatic, temperature and topological variables inherent in DNA-binding resin isolation procedures. Since the present invention requires only aqueous solutions of relatively inexpensive chemicals, the cost is significantly less than other methods. Perhaps the greatest benefit of the present invention is that the extra-chromosomal DNA is isolated in an undegraded and intact condition, providing a more useful final product.

**Brief Description of the Invention**

The present invention provides methods and materials for isolating substantially pure and undegraded extra-chromosomal nucleic acids from biological materials. The solution contains effective amounts of a chaotropic agent, a buffer present in an amount sufficient to maintain an alkaline pH equal to or greater than about 7.5, salt, detergent, and alcohol. The invention is also a method for isolating substantially pure and undegraded extra-chromosomal DNA from biological materials. The steps in the method include precipitating extra-chromosomal DNA and contaminating RNA from a cell lysate, treating the extra-chromosomal DNA with RNase to digest the contaminating RNA, removing residual contaminants from extra-chromosomal DNA by adding the chaotropic solution, precipitating the DNA with an alcohol, pelleting the DNA by sedimentation, and washing the sedimented DNA with alcohol. An alternative use of the invention is for recovery of any type of DNA from agarose gel preparations.

**Detailed Description of the Invention**

The following terms have the following meanings for the purposes of this application, unless expressly stated to the contrary herein.

By "lysis" is meant cellular dissociation involving physical disruption and breakage of the cell wall and/or membrane, causing intracellular components including nucleic acids to be released into the surrounding medium.

By "incomplete lysis" is meant the lysis of a cell or a group of cells which renders the cell chromosomes, cell membranes and other components capable of forming a precipitate while leaving the extracellular DNA in solution.

By "alkaline lysis" is meant the breaking open of a cell or a group of cells, or the liberation of some or all of the intracellular components following treatment of the cell or cells with an alkali or base which partially digests the organism's cell wall or membrane. This type of lysis breaks open a cell or a group of cells, and renders the cell chromosomes, cell membranes and other components capable of forming a precipitate while leaving the extracellular DNA in solution.

By "enzymatic lysis" is meant the breaking open of a cell or a group of cells, or the liberation of some or all of the intracellular components following treatment of the cell or cells with an enzyme which partially digests the organism's cell wall. This type of lysis breaks open a cell or a group of cells, and renders the cell chromosomes, cell membranes and other components capable of forming a precipitate while leaving the extracellular DNA in solution.

By "detergent" or "surfactant" is meant a molecule or class of molecules which have a hydrophobic region or moiety capable of interacting with hydrophobic solvents and the hydrophobic portions of cellular membranes, and a hydrophilic

region or moiety which may have a positive or a negative charge in solution, or alternately may have a polar region with no charge at all.

Reference to the "release of nucleic acids" is intended to mean the liberation of nucleic acids in sufficient quantities such that the method of release is useful for further purification and subsequent use in molecular biology.

By "nucleic acid" or "nucleic acids" is meant polydeoxyribonucleotides or polyribonucleotides of at least two, and preferably 10 or more nucleotides in length. The term "nucleic acid" includes polynucleotides, oligonucleotides, and DNA or RNA molecules. The term "nucleic acid" can refer to either single-stranded or double-stranded polynucleotides, or both.

By "biological sample" is meant any specimen or sample containing substances of biological or biochemical origin. "Biological materials" include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA or BACs; yeast transformed with yeast expression vectors or YACs; insect cell systems infected with viral expression vectors (*e.g.* baculovirus); plant cell systems transformed with viral expression vectors (*e.g.* cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV), or cells derived from higher organisms such as mammalian tissue culture cells transfected with recombinant plasmid DNA or infected with viral expression vectors. "Biological material" may also refer to agarose or polyacrilamide gel preparations used to separate any nucleic acids by size, charge or other characteristic.

As used herein, "substantially purified" refers to recovery of nucleic acid which is at least 80% and preferably 90-95% purified with respect to removal of a contaminant, *e.g.*, cellular components such as protein, lipid or salt; thus, the term

"substantially purified" generally refers to separation of a majority of cellular proteins or reaction contaminants from the sample, so that compounds capable of interfering with the subsequent use of the isolated nucleic acid are removed.

As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components.

As used herein, "chaotropic environment" refers to an environment which contains appropriate chaotropic agents, such as urea in sufficient concentration to disrupt the tertiary structure of proteins, or which is maintained at a temperature or other condition which causes such disruption.

Chaotropic agents or conditions such as temperature and pH may disrupt structure in a variety of ways, including the disruption of hydrogen bonds. Suitable chaotropic environments include 2-8M urea, 4-7M guanidinium, detergents such as SDS at concentrations around 0.1% by weight, and acids such as acetic acid at concentrations of about 1M, basic conditions of, *e.g.*, pH 11 and above, and elevated temperatures. When placed in a chaotropic environment, the normal physiological conformation of proteins may be reversibly as well as irreversibly altered, and the primary structure may be "unfolded" to varying degrees, depending on the concentration of the chaotropic agent and the degree of severity of other chaotropic conditions. It should be understood that agents and/or conditions that create chaotropic environments can be used in combination or in sequence. For example, mixtures of chaotropic agents can be used, or the biological sample may first be placed in a chaotropic environment created by one chaotropic agent, and then subjected to a second chaotropic environment created by another agent or by temperature.

The products and methods of the present invention provide a highly efficient, simple means of isolating DNA from a single biological sample, such as bacteria. Advantageously, these results can be achieved without the use of toxic or caustic reagents, DNA-binding matrices, or the use of expensive ultracentrifugation equipment.

In its broadest aspects, the invention encompasses solution and methods for isolating substantially pure and undegraded extra-chromosomal nucleic acids from biological materials.

The present invention is an entirely liquid based system that will not require the production or use of columns or beads employing DNA-binding materials. No toxic chemicals such as phenol or chloroform will be needed. The use of the present invention to isolate plasmid or other extra-chromosomal DNA is significantly less expensive and as fast or faster than methods currently known and available. The methods can be adapted to differing amounts of biological materials by changing volumes of the reagents used. For example, plasmid DNA can be harvested from bacterial cultures in what are conventionally known as "mini-", "midi-", or "maxi-prep" amounts. The extra-chromosomal DNA isolated using the materials and methods of this invention is of particularly high purity, quality, and thus, usefulness.

The present invention is distinct from the prior art disclosures of chaotropic agents used to purify DNA because those materials and methods were designed for the isolation of genomic DNA. As such they use a formulation and method that will disrupt cell membranes and release both genomic and any extra-chromosomal DNA into a heterogeneous mixture. Isolation of extra-chromosomal DNA from such a mixture is, at best, an impractical and highly inefficient process.

The present invention is also distinct from those in the prior art that are directed to the isolation of extrachromosomal DNA. In DNA-binding matrix methods and conventional methods, cells or bacteria are first lysed using a standard alkaline lysis method. Alkaline lysis releases extrachromosomal-DNA and RNA from the cells or bacteria into the crude lysate supernatant, but genomic DNA remains membrane-bound and is pelleted in the bacterial debris along with the membranes with centrifugation. RNA is digested by adding RNase to the lysate. In methods using DNA-binding matrices, the RNase-treated lysate is applied to column or beads containing the DNA-binding materials, washed to remove protein contaminants, and eluted. In conventional methods, the DNA in a lysate is separated from contaminants first by ultracentrifugation on a gradient, such as cesium salts, and then dialyzed and/or purified with a series of phenol:chloroform extractions.

In the present invention, a lysate is first prepared using a standard alkaline lysis method. The neutralized solution is treated with an alcohol precipitation followed by RNase to degrade contaminating RNA in the lysate. Next, a chaotropic solution is added to the lysate to remove residual protein and degraded RNA contaminants from the extra-chromosomal DNA.

The steps of the present methods are described more fully as follows:

1. **Cell lysis**

As already mentioned a typical method of the present invention involves the recovery of purified plasmid from a bacterial lysate. Accordingly, typical purification procedures first require the preparation of the bacterial lysate from a bacterial culture. Those skilled in the art are familiar with methods for lysing bacteria. The present

invention is described in terms of the known alkaline lysis procedure, however, any procedure which effectively lysis the bacterial cells and releases plasmid DNA is applicable, such as enzymatic lysis. Typically these procedures involve adding a bacterial pellet to solutions which break open the bacterial cells, and cause the bacterial chromosomes, cell membranes and other components to form a precipitate while leaving the plasmid DNA in solution.

The alkaline lysis is generally prepared by pelleting a suspension of a biological material, such as a liquid culture of bacteria, through centrifugation. The supernatant is poured off and the pellet resuspended in buffer solution. The biological material is then lysed by addition of a strong base, *e.g.*, sodium hydroxide, in the presence of a detergent such as sodium dodecyl sulfate (SDS). The alkaline lysis is terminated by the addition of a neutrilzation solution such as sodium or potassium acetate. The neutralized lysis mixture is centrifuged to pellet all cellular debris, including membranes and membrane-bound chromosomal DNA. The lysate can alternatively be separated from the cellular debris by various filtration methods well known in the art, e.g., by gravity or by vacuum. The supernatant ("lysate") is poured into a clean tube for the precipitation step.

**2. Precipitation and RNase treatment**

Alcohol, such as ethanol, is added to the lysate for a final concentration of about 70%. After mixing thoroughly, the solution is centrifuged to pellet the nucleic acids, which will include RNA and extra-chromosomal DNA. The supernatant is discarded. The pellet is resuspended in a buffer solution containing RNase to degrade all RNA contaminants. The concentration of the buffer solution is generally an amount such that the pH is from about 7 to about 12, preferably where the pH is from

about 7 to about 10, and most preferably where the pH is from about 7 to about 8. The RNase incubation is generally performed at 37° (but can be performed at room temperature) for about 10 minutes.

**3. Chaotropic Solution Treatment**

The RNase-treated solution is then mixed with a chaotropic solution and stored at about room temperature for about 10 minutes. The mixture of the RNase-treated solution and the chaotropic solution is then treated with a solvent (0.3 to 1.0 of solution volume) in order to precipitate the extra-chromosomal DNA, and subsequently centrifuged to pellet the DNA away from contaminating proteins and degraded RNA. The supernatant is removed and discarded. The DNA pellet is washed with a solvent (about 60% to about 80%) to remove any residual traces of the chaotropic solution. Following centrifugation, the supernatant is removed and the DNA pellet is allowed to dry. The DNA pellet is then dissolved in water or any suitable buffer.

For the methods of the present invention, the chaotropic solution is generally an aqueous mixture of effective amounts of one or more chaotropic agents. Any chaotropic agent is useful in the practice of the present invention including but not limited to guanidine thiocyanate (GnSCN), sodium iodide, sodium perchlorate, guanidine hydrochloride, urea, hydroxides such as sodium or potassium hydroxide, guanidine salt, potassium thiocyanate, formamide, and sodium chloride. Chaotropic agents include a combination of these reagents, such as a mixture of base with urea or guanidine hydrochloride.

Preferred embodiments of the present invention use aqueous solutions of 6 M guanidine hydrochloride. Best results are achieved when the chaotropic agent is

present in relatively high concentrations ranging from 1 M to 12 M. Preferred chaotropic agents for the solution include guanidinc thiocyante, guanidine hydrochloride, sodium iodide, and mixtures thereof. The concentration of chaotropes in the solution is preferably in the range of from about 1 M to about 7 M and is more preferably in the range of from about 2 M to about 5 M.

The chaotropic solution generally comprises one or more chaotropic agents and optionally contains one or more compounds selected from the group consisting of buffers or buffer solutions, solvents, surfactants, salts, preservatives, anti-oxidants, bacteriostats, solutes, reducing agents, stabilizers and mixtures thereof.

Representative acceptable buffers comprise nontoxic buffer and solution thereof, solutions that resist change of pH thereby giving stability to the components present in the solution. In another embodiment the buffer and solutions thereof can be used alone and, in a presently preferred embodiment, the buffer and solution thereof is used in combination with acceptable aqueous miscible fluids. Typical buffer aqueous solutions comprise sodium dihydrogen phosphate and disodium monohydrogen phosphate; sodium dihydrogen phosphate , disodium monohydrogen phosphate and sodium chloride ; sodium carbonate , sodium monohydrogen carbonate and sodium chloride ; potassium dihydrogen phosphate and sodium monohydrogen phosphate; potassium dihydrogen phosphate and sodium monohydrogen phosphate; potassium hydrogen tartrate and potassium dihydrogen phosphate ; acetic acid and sodium acetate , and the like. The buffers comprise also citric acid and sodium hydroxide ; potassium hydrogen phthatate and sodium hydroxide; potassium hydrogen phosphate and sodium phosphate; tris(hydroxymethyl) aminomethane and hydrochloric acid; sodium tetraborate and hydrochloric acid; glycine and hydrochloric acid;

triethanolamine and hydrochloric acid; N-tris (hydroxymethyl)methyl-2-amino sulfonic acid and sodium hydroxide, and the like. The buffer aqueous solution in another embodiment can comprise a sole component such as sodium phosphate monobasic, sodium phosphate dibasic, potassium hydrogen tartrate, potassium dihydrogen citrate, potassium hydrogen phthalate, sodium tetraborate, sodium carbonate, sodium hydrogen carbonate and mixtures thereof.

As used herein, "solvent" refers to alcohols and polar aprotic solvents. Alcohols are meant in the sense of the commonly used terminology for alcohol, preferably lower alcohols with 1 to 10 carbon atoms, more preferably methanol, ethanol, iso-propanol, n-propanol, or t-butanol, as well as glycerol, propylene glycol, ethylene glycol, polypropylene glycol, and polyethylene glycol, and most preferably ethanol or iso-propanol. Such alcohols are solvents that, when added to aqueous solution, increase the hydrophobicity of the solution by decreasing solution polarity. Polar aprotic solvents are such molecules as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), N-methylpyrrolidone (NMP), tetrahydrofuran (THF), dioxane, acetonitrile, etc., that can be used in place of or in addition to the alcohol. Preferably a lower alcohol such as ethanol, propanol or butanol, is also included, at from about 5% to about 40% by volume and more preferably at from about 15% to about 20% by volume.

Representative acceptable surfactants for the present purpose comprise anionic, cationic, amphoteric and nonionic surfactants. More specific examples of surfactants comprise sorbitan trioleate, sorbitan tristearate, propylene glycol monostearate; sorbitan sesquiolate; glycerol monostearate; sorbitan monooleate; propylene glycol monolaurate; sorbitan monostearate; diethylene glycol

monostearate; glycerol monostearate; diethylmonolaurate; sorbitan monopalmitate; sorbitan monolaurate; TRITONS; polyoxyethylene ethers; polyoxyethylene lauryl ether; polyoxyethylene sorbitan monostearate; polyoxyethylene sorbitan monooleate; polyoxyethylene sorbitan tristearate; polyoxyethylene sorbitan trioleate; polyoxyethylene glycol monooleate; polyoxyethylene glycol monostearate; triethanolamine oleate; polyoxyethylene monyl phenol; polyethylene glycol monolaurate; polyoxyethylene sorbitan monolaurate; polyoxyethylene sorbitan monostearate; polyoxyethylene sorbitan monooleate; polyoxyethylene stearyl ether; polyoxyethylene oleyl ether; polyoxyethylene sorbitan monopalmitate; polyoxyethylene cetyl ether; polyoxyethylene stearate; sodium oleate; potassium oleate; cetyl ethyl morpholinium ethosulfate; sodium lauryl sulfate; sodium caprylate; sodium caprate; sodium laurate; sodium myristate; sodium cholate; sodium desoxycholate; sodium dihydrocholate; tetradecyltrimethyl ammonium bromide; hexadecylpyridinium chloride; Tween 20; Tween 30; Tween 80, and mixtures thereof.

A compatible surfactant is generally included in an amount sufficient to promote solubilization of contaminating proteins, but at low enough concentration so that the detergent stays in solution. The preferred surfactants are non-ionic detergents such as the octoxynols and polysorbates. Examples of these are detergents with the trade names of TRITON X-100, TRITON X-114, NP 40, CONCO-NIX-100, IGEPAL CA-630, AND NEUTRONYX 605. The amount of surfactant used is preferably about 0.001% to 7.5%.

Acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulfonic, tartaric, citric, methane sulfonic, formic, malonic,

succinic, naphthalene-2-sulfonic, and benzene sulfonic. Also, acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium, lithium or calcium salts (potassium chloride, sodium acetate, sodium nitrite, lithium chloride, and sodium bromide.). The salt concentration is generally in the range of about 0.01 M to about 1 M and preferably in the range of about 0.1 M to about 0.5 M.

As used herein, "reducing agent" refers to a compound that, in a suitable concentration in aqueous solution, maintains sulfhydryl groups so that the intra- or intermolecular disulfide bonds are chemically disrupted. Representative examples of suitable reducing agents include dithiothreitol (DTT), dithioerythritol (DTE), beta-mercaptoethanol (BME), cysteine, cysteamine, thioglycolate, glutathione, and sodium borohydride. While emphasis is placed on thiol-containing compounds, any material that is capable of the disulfide to thiol conversion without undesirable side reactions is included in this definition.

In another preferred embodiment, at least one antioxidant is added to the solutions of the present invention. The antioxidants are preferably BHA, BHT, octyl or dodecyl gallate, $SO_2$, for instance in the form of sodium sulfites or sodium thiosulfite, lactic acid, citric acid, tartaric acid and/or the salts thereof, vitamin C, vitamin E and uric acid and the salts thereof. Especially preferred, physiologically tolerated antioxidants are alpha -tocopherol, bilirubin, vitamin C, vitamin E, uric acid, and the salts and/or derivatives thereof. Uric acid and the salts thereof are preferably added in a concentration of from about 0.01 to about 1 wt. %, vitamin C in a concentration of 0 from about 0.01 to 2 wt. %, and vitamin E in a concentration of from about 0.01 to about 0.1 wt. %.

The preservatives used may be formic acid in a concentration of preferably from about 0.03 to about 0.4 wt. %, acetic acid (more preferably from about 0.3 to about 3 wt. %), propionic acid, lactic acid and sorbic acid in a concentration of preferably 0.05 to 6 wt. %, especially preferred is from about 0.05 to about 12 wt. %, $SO_2$ (preferably from about 0.01 to about 0.6 wt. %), salicylic acid and the salts thereof (preferably from about 0.01 to about 0.5 wt. %), PBH ester (preferably from about 0.05 to about 0.6 wt. %), imidazolidinyl urea derivatives (preferably from about 0.01 to about 0.6 wt. %), chlorohexidine, NIPA-ESTER Registered TM or antibiotics.

Sterilization can be accomplished by any art-recognized technique, including but not limited to, filtration or addition of antibacterial or antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid or thimerosal. Further, isotonic agents such as sugars or sodium chloride may be incorporated in the subject compositions.

**4. An alternative use for the invention**

Extra-chromosomal DNA or fragments of genomic DNA can be partially purified by conventional agarose or acrylamide gel electrophoresis on the basis of size, charge, or other properties. Since these materials are unlikely to contain RNA contaminants, no RNase treatment steps are needed. The region of the gel containing the DNA of interest is excised and solublized using an enzyme that specifically cleaves the gel matriux bonds without damaging DNA, *e.g.*, beta-agarose. After solublization, the gel and DNA solution can be treated with the chaotropic solution of the present invention to remove gel components and other contaminants. The chaotropic solution is added to the DNA/gel solution at a ratio of about 10:1, and stored at about room

temperature for about 10 minutes. An alcohol (from about 0.3 to about 1.0 volumes relative to volume of the mixture of chaotropic solution and the DNA/gel solution) is added to the mixture in order to promote precipitation of the DNA. This mixture is centrifuged to pellet the DNA. The supernatant is removed and discarded. The DNA pellet is washed with ethanol or 2-propanol to remove any residual traces of the chaotropic solution. The effective final concentration of alcohol is from about 60% to about 80%. Following centrifugation, the supernatant is removed and the DNA pellet is allowed to dry. The DNA pellet is then dissolved in water or any suitable buffer.

**Reagent Kits**

The present invention also contemplates a reagent system, typically in kit form, that can be utilized in carrying out the before-described isolation methods. The system includes, in an amount sufficient for at least one isolation, a separately packaged reagent for a process for the isolation of extra-chromosomal nucleic acids in a biological sample. Instructions for use of the packaged reagent are also typically included.

As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil and the like capable of holding within fixed limits reagents of the present invention.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like. The packaging materials discussed herein in relation to diagnostic systems are those

customarily utilized in diagnostic systems. Such materials include glass and plastic (*e.g.*, polyethylene, polypropylene and polycarbonate) bottles, vials, plastic and plastic-foil laminated envelopes and the like.

Specifically, the invention provides a compartmentalized kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the chaotropic agents of the present invention; and (b) one or more other containers comprising one or more of the following: wash reagents, lysis reagents, Rnase, Rnase digestion buffer, and polyacrylamide or agarose-gel solubilization solution.

In detail, a compartmentalized kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the chaotropic agent used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to perform lysis of cells, *e.g.*, RNase digestion solution, *etc.*

In the following examples of the present invention, all temperatures are given on the Centigrade scale, and percentages are grams (for solids) or milliliters (for liquids) per 100 ml of water.

**Example 1**

1. Prepare a 100 ml culture of *E. coli* harboring the plasmid DNA of interest. Suitable culture media include sterile preparations of LB or 2XYT broth, which are widely known and used in the propagation of bacteria. Typically, 100 ml of broth is placed in a sterile 500 ml culture flask. The broth is innoculated with *E. coli* harboring the plasmid of interest, and the flask is placed in a shaking incubator overnight at 37°. The following day, the culture is transferred to a centrifuge tube and the bacteria are pelleted by centifuging for 5 minutes at about 1900 g. This can be done in a SORVAL™ RC5B model centrifuge, using an SLA-1000 rotor at 4000 rpm.
2. Decant the supernatant and place the tube containing the pelleted bacteria in an ice bath.
3. Resuspend the bacterial pellet in 4 ml of a solution containing 50 mM Tris-HCl pH 8.0 and 10 mM EDTA, by pipeting up and down in a 10 ml serological pipet.
4. When bacterial pellet is evenly suspended, add 8 ml of a solution containing 200 mM NaOH and 1% SDS. Gently mix by inverting the capped tube 15-30 times. Care must be taken not to shake the mixture. Store on ice 5 minutes.
5. Add 6 ml of a solution containing 3 M potassium acetate pH 5.5. Gently mix by inverting the tube 15-30 times. Care must be taken to not shake the mixture. Store on ice for 10 minutes.
6. Separate plasmid DNA from contaminating chromosomal DNA and bacterial debris by centrifuging at 22000 g for 20 minutes at 4°. This can be done in a SORVAL™ RC5B model centrifuge, using an SS34 rotor at 13,500 rpm.

7. Transfer the supernatant to a clean 250 ml centrifuge bottle and add 40 ml ice-cold 95% ethanol. Vigorously swirl mixture by hand, and store on ice for 10 minutes.

8. Centrifuge at about 11000 g for 15 minutes at 4°. This can be done in a SORVAL™ RC5B model centrifuge, using an SLA-1000 rotor at 9,000 rpm.

9. Decant liquid. Close observation of the tube will reveal the location of a large white pellet. This is the crude isolate of nucleic acids, containing plasmid DNA, bacterial RNA, and residual protein contaminants. Carefully pipet away any excess liquid in the tube, but do not disturb the pellet.

10. Leave the cap off the tube and allow the pellet to air dry for 5-10 minutes.

11. Resuspend pellets in 1 ml of a solution containing 10 mM Tris-HCl pH 7.5 and 50 ug/ml RNase A.. Transfer the solution to a 1.5 ml microcentrifuge tube such as an Eppendorf™ tube. Incubate in a water bath at 37° for 10 minutes.

12. Spin down excess debris. Place the microcentrifuge tube in a microcentrifuge at maximum speed (typically 12,000-15,000 rpm) for 5-10 minutes at 4°.

13. Transfer the supernatant to a clean 30 ml centrifuge tube. Add 10 ml of a chaotropic solution containing 4M guanadine isothiocyanate, 0.05 M TRIZMA Base pH 9.2, 0.3 M sodium acetate, 0.1% TRITON X-114, and 17% 2-propanol. Mix by VORTEX® agitation for 5-10 seconds, and store at room temperature for 10 minutes.

14. Add 7 ml of ice-cold 95% ethanol, mix by VORTEX® agitation 5-10 seconds, and store on ice for 10 minutes.

15. Centrifuge at about 27000 g for 20 minutes at 4°. This can be done in a SORVAL™ RC5B model centrifuge, using an SS34 rotor at 15,000 rpm.

16. Decant the supernantant. Wash the pellet once by adding about 5 ml of 70% ethanol so that the liquid covers the pellet, mixing by VORTEX™ agitation for 10-20 seconds, and then recentrifuging in an SS34 rotor at 15,000 rpm for 10-15 minutes.
17. Use a micropipeter to carefully remove as much supernatant as possible. Leave the cap of the centrifuge tube open, and allow the DNA pellet to air dry. The pellet is usually dry after about 10-30 minutes.
18. Dissolve the DNA pellet by adding 500 µl 10 mM Tris, pH 7.8 to the centrifuge tube, and transfer the dissolved DNA to a 1.5 ml Eppendorf™ tube.
19. Place the tube in a 55° heat block for 10 minutes to promote solubilization. Agitate the solution briefly using a VORTEX™ mixer.
20. Spin for 10 minutes at maximum speed in a microcentrifuge at 4° to remove any residual contaminants from the DNA solution.
21. Transfer DNA solution to a clean 1.5 ml microcentrifuge tube for long-term storage.

**Example 2**

1. Prepare a lysate from bacteria harboring the extra-chromosomal DNA of interest. This can be done following the protocol outlined on pp 1.21-1.52 *of Molecular Cloning, A Laboratory Manual* Second Edition. Sambrook, J.; Fritsch, E.F.; Maniatis, T. Cold Spring Harbor Laboratory Press, 1989.
2. Transfer the lysate to a centrifuge tube which can hold a volume equal to or greater than 3.5 times the volume of lysate. Add 2.5 parts of 95% ethanol to 1 part lysate. Swirl vigorously by hand to mix thouroughly.

3. Centrifuge at about 21000 g for 20 minutes to the nucleic acid pellet. This can be done in a SORVAL™ RC5B model centrifuge, using an SS34 rotor at 13,500 rpm. Decant supernatant and allow the pellet to air dry.
4. Resuspend the pellet in a solution of 10 mM Tris-HCl, pH 7.5 in the presence of RNase A (50 ug/ml) and transfer to an 1.5 ml microfuge tube. Incubate the lysate at 37° heat block for 10 minutes. Sediment contaminating material by centrifuging the microfuge tube at maximum speed in a microcentrifuge for 10 minutes at 4°.
5. Transfer the supernatant to a clean 30 ml centrifuge tube. Treat the supernatant with 10 ml of a chaotropic solution containing 5 M guanadine isothiocyanate, 0.05 M TRIZMA Base pH 9.2, 0.1 M sodium acetate, 0.1% NP-40, and 15 % 2-propanol. Mix by VORTEX® agitation for 5-10 seconds, and store at room temperature for 10 minutes.
6. Add 7 ml of ice-cold 95% ethanol, mix by VORTEX™ agitation 5-10 seconds, and store on ice for 10 minutes.
7. Centrifuge at about 27000 g for 20 minutes at 4°. This can be done in a SORVAL™ RC5B model centrifuge, using an SS34 rotor at 15,000 rpm.
8. Decant the supernantant. Wash the pellet once by adding about 5 ml of 70% ethanol so that the liquid covers the pellet, mixing by VORTEX™ agitation for 10-20 seconds, and then recentrifuging in a microcentrifuge at maximum speed for 10-15 minutes.
9. Use a micropipeter to carefully remove as much supernatant as possible. Leave the cap of the microcentrifuge tube open, and allow the DNA pellet to air dry. The pellet is usually dry after about 10-30 minutes.

10. Dissolve the DNA pellet by adding 500 µl 10 mM Tris, pH 7.8 to the microcentrifuge tube.

11. Place the tube in a 55° heat block for 10 minutes to promote solubilization. Agitate the solution briefly using a VORTEX™ mixer.

12. Spin for 10 minutes at maximum speed in a microcentrifuge at 4° to remove any residual contaminants from the DNA solution.

13. Transfer DNA solution to a clean 1.5 ml microcentrifuge tube for long-term storage.

**Example 3**

Prepare a lysate from 500 ml of bacterial culture harboring the BAC DNA of interest. This can be done following the protocol outlined on pp 1.21-1.52 *of Molecular Cloning, A Laboratory Manual* Second Edition. Sambrook, J.; Fritsch, E.F.; Maniatis, T. Cold Spring Harbor Laboratory Press, 1989. Because only one copy of a BAC will be present in each bacterium, as opposed to plasmid DNA with anywhere from 20-300 copies per bacterium, much larger culture volumes is required to isolate a useful amount of DNA. While the amounts of reagents needed for the alkaline lysis and neutralization steps will be proportionately increased, the precipitated pellet obtained from the lysate is resuspended in about 1-2 ml of 10 mM Tris-HCl pH 7.5 buffer for the RNase treatment step.

1. Transfer the lysate to a centrifuge tube that can hold a volume equal to or greater than 3.5 times the volume of lysate. Add 2.5 parts of 95% ethanol to 1 part lysate. Swirl vigorously by hand to mix thoroughly.

2. Centrifuge at about 11000 g for 15 minutes to pellet crude nucleic acids. This can be done in a SORVAL™ RC5B model centrifuge, using an SLA-1000 rotor at 9,000 rpm. Decant supernatant.

3. Resuspend the pellet in a solution of 10 mM Tris-HCl, pH 7.5 containing RNase A and transfer to one or several 1.5 ml microfuge tubes. Because a greater amount of culture volume is needed to isolate BAC DNA, there will be a corresponding increase in the amount of RNA contamination. Add RNase A to the lysate such that the final concentration of RNase A is 200 ug/ml. Incubate the lysate at 37° on a heat block for 1 hour. Pellet contaminating material away from the extra-chromosomal DNA by centrifuging at maximum speed in a microcentrifuge at 4°.

4. Transfer the RNase-treated supernatant to a 30 ml centrifuge tube. Treat the supernatant with 10 ml of a chaotropic solution containing 4 M guanadine isothiocyanate, 0.05 M TRIZMA Base pH 10, 0.5 M sodium acetate, 0.45% TRITON X-100, and 20% ethanol. Mix by VORTEX™ agitation for 5-10 seconds, and store at room temperature for 10 minutes.

5. Add 7 ml of ice-cold 95% ethanol, mix by VORTEX ™ agitation 5-10 seconds, and store on ice for 10 minutes.

6. Centrifuge at about 27000 g for 20 minutes to pellet crude DNA. This can be done in a SORVAL™ RC5B model centrifuge, using an SS34 rotor at 15.000 rpm.

7. Decant the supernantant. Wash the pellet once by adding about 5 ml of 70% ethanol so that the liquid covers the pellet, mixing by VORTEX™ agitation for 10-20 seconds, and then recentrifuging for 10-15 minutes.

8. Use a micropipeter to carefully remove as much supernatant as possible. Leave the cap of the centrifuge tube open, and allow the DNA pellet to air dry. The pellet is usually dry after about 10-30 minutes.
9. Dissolve the DNA pellet by adding 500 µl 10 mM Tris, pH 7.8 and transfer the dissolved DNA to a microcentrifuge tube.
10. Place the tube in a 55° heat block for 10 minutes to promote solubilization. Agitate the solution briefly using a VORTEX ® mixer.
11. Spin for 10 minutes at maximum speed in a microcentrifuge at 4° to remove any residual contaminants from the DNA solution.
12. Transfer DNA solution to a clean 1.5 ml microcentrifuge tube for long-term storage.

Example 4

1. Prepare a 30 ml culture of *E. coli* harboring the plasmid DNA of interest. Suitable culture media include sterile preparations of LB or 2XYT broth, which are widely known and used in the propagation of bacteria. Typically, 30 ml of broth is placed in a sterile 150 ml culture flask. The broth is inoculated with *E. coli* harboring the plasmid of interest, and the flask is placed in a shaking incubator overnight at 37°. The following day, the culture is transferred to a 50 ml FALCON® tube and the bacteria are pelleted by centifuging for 5 minutes at 1500 g. This can be done in a Beckman centrifuge, using an JA20 rotor at 2000 rpm.
2. Decant the supernatant and place the tube containing the pelleted bacteria in an ice bath.

3. Resuspend the bacterial pellet in 2 ml of a solution containing 50 mM Tris-HCl pH 8.0 and 10 mM EDTA, by pipeting up and down in a 10 serological pipet.
4. When bacterial pellet is evenly suspended, add 4 ml of a solution containing 200 mM NaOH and 1% SDS. Gently mix by inverting the capped tube 15-30 times. Care must be taken to not shake the mixture. Store on ice 5 minutes.
5. Add 3 ml of a solution containing 3 M potassium acetate pH 5.5. Gently mix by inverting the tube 15-30 times. Care must be taken to not shake the mixture. Store on ice for 10 minutes.
6. Separate plasmid DNA from contaminating chromosomal DNA and bacterial debris by centrifuging at about 8000g for 30 minutes at 4°. This can be done in a Beckman centrifuge, using an JA20 rotor at 10,000 rpm.
7. Transfer the supernatant to a clean 50 ml FALCON tube and add 25 ml ice-cold 95% ethanol. Vigorously swirl mixture by hand, and store on ice for 10 minutes.
8. Centrifuge at 8000 g for 30 minutes at 4°. This can be done in a Beckman centrifuge, using an JA20 rotor at 10,000 rpm.
9. Decant liquid. Close observation of the tube will reveal the location of a small white pellet. This is the crude isolate of nucleic acids, containing plasmid DNA, bacterial RNA, and residual protein contaminants. Carefully pipet away any excess liquid in the tube, but do not disturb the pellet.
10. Leave the cap off the tube and allow the pellet to air dry for 5-10 minutes.
11. Resuspend pellet in 1 ml of a solution containing 10 mM Tris-HCl pH 7.5 and 50 ug/ml RNase A.. Transfer the solution to a 1.5 ml microcentrifuge tube such as an Eppendorf™ tube. Incubate in a water bath at 37° for 10 minutes.

12. Spin down excess debris. Place the microcentrifuge tube in a microcentrifuge at maximum speed (typically 12,000-15,000 rpm) for 5-10 minutes at 4°.

13. Transfer the supernatant to a clean 50 ml FALCON™ tube. Add 5 ml of a chaotropic solution containing 3 M guanadine isothiocyanate, 0.1 M guanidine hydrochloride, 0.05 M TRIZMA Base pH 8.5, 0.2 M sodium acetate, 0.5% TRITON X-100, and 17% 2-propanol. Mix by VORTEX™ agitation for 5-10 seconds, and store at room temperature for 10 minutes.

14. Add 3.5 ml of ice-cold 95% ethanol, mix by VORTEX ™ agitation 5-10 seconds, and store on ice for 10 minutes.

15. Centrifuge at 8000 g for 30 minutes at 4°. This can be done in a Beckman centrifuge, using an JA20 rotor at 10,000 rpm.

16. Decant the supernantant. Wash the pellet once by adding about 3 ml of 70% ethanol so that the liquid covers the pellet, mixing by VORTEX ™ agitation for 10-20 seconds, and then recentrifuge for 10-15 minutes.

17. Use a micropipeter to carefully remove as much supernatant as possible. Leave the cap of the 50 ml FALCON™ tube open, and allow the DNA pellet to air dry. The pellet is usually dry after about 20-30 minutes.

18. Dissolve the DNA pellet by adding 500 µl 10 mM Tris, pH 7.8 and transfer the dissolved DNA to a microcentrifuge tube.

19. Place the tube in a 55° heat block for 10 minutes to promote solubilization. Agitate the solution briefly using a VORTEX™ mixer.

20. Spin for 10 minutes at maximum speed in a microcentrifuge at 4° to remove any residual contaminants from the DNA solution.

21. Transfer DNA solution to a clean 1.5 ml microcentrifuge tube for long-term storage.

**Example 5.**

1. Prepare a 2 ml culture of *E. coli* harboring the plasmid DNA of interest. Suitable culture media include sterile preparations of LB or 2XYT broth, which are widely known and used in the propagation of bacteria. Typically, 2 ml of broth is placed in a sterile 5 ml culture tube. The broth is innoculated with *E. coli* harboring the plasmid of interest, and the flask is placed in a shaking incubator overnight at 37°. The following day, the bacteria are pelleted by centifuging for 2 minutes at maximum speed (12000-15000 rpm) in a microcentrifuge at room temperature.
2. Decant the supernatant and place the tube containing the pelleted bacteria in an ice bath.
3. Resuspend the bacterial pellet in 100 µl of a solution containing 50 mM Tris-HCl pH 8.0 and 10 mM EDTA.
4. When bacterial pellet is evenly suspended, add 200 µl of a solution containing 200 mM NaOH and 1% SDS. Gently mix by inverting the capped tube 15-30 times. Care must be taken to not shake the mixture. Store on ice 5 minutes.
5. Add 200 µl of a solution containing 3 M potassium acetate pH 5.5. Gently mix by inverting the tube 15-30 times. Care must be taken to not shake the mixture. Store on ice for 10 minutes.
6. Separate plasmid DNA from contaminating chromosomal DNA and bacterial debris by centrifuging at maximum speed (12000-15000 rpm) in a microcentrifuge at 4° for about 10 minutes.

7. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube and add 900 µl ice-cold 95% ethanol. Mix vigorously, and store on ice for 10 minutes.
8. Centrifuge at maximum speed (12000-15000 rpm) in a microcentrifuge at 4° for about 20 minutes
9. Decant liquid. Close observation of the tube will reveal the location of a small white pellet. This is the crude isolate of nucleic acids, containing plasmid DNA, bacterial RNA, and residual protein contaminants. Carefully pipet away any excess liquid in the tube, but do not disturb the pellet.
10. Leave the cap off the tube and allow the pellet to air dry for 5-10 minutes.
11. Resuspend pellet in 50 µl of a solution containing 10 mM Tris-HCl pH 7.5 and 50 ug/ml RNase A. Incubate in a water bath at 37° for 10 minutes.
12. Spin down excess debris. Place the microcentrifuge tube in a microcentrifuge at maximum speed (typically 12,000-15,000 rpm) for 5-10 minutes at 4°.
13. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube and add 700 µl of a chaotropic solution containing 3M guanadine isothiocyanate, 0.05 M TRIZMA Base pH 9.0, 0.4 M sodium acetate, 0.3% TRITON X-100, and 17% ethanol. Mix by VORTEX agitation for 5-10 seconds, and store at room temperature for 10 minutes.
14. Add 350 µl of ice-cold 95% ethanol, mix by VORTEX agitation 5-10 seconds, and store on ice for 10 minutes
15. Place the microcentrifuge tube in a microcentrifuge at maximum speed (typically 12,000-15,000 rpm) for 5-10 minutes at 4°.
16. Decant the supernantant. Wash the pellet once by adding about 300 µl of 70% ethanol so that the liquid covers the pellet, mixing by VORTEX agitation for 10-

20 seconds, and then recentrifuging in a microcentrifuge at maximum speed for 10-15 minutes.

17. Use a micropipeter to carefully remove as much supernatant as possible. Leave the cap of the microcentrifuge tube open, and allow the DNA pellet to air dry. The pellet is usually dry after about 10-30 minutes.

18. Dissolve the DNA pellet by adding 90 µl 10 mM Tris, pH 7.8 to the microcentrifuge tube.

19. Place the tube in a 55° heat block for 10 minutes to promote solubilization. Agitate the solution briefly using a VORTEX mixer.

20. Spin for 10 minutes at maximum speed in a microcentrifuge at 4° to remove any residual contaminants from the DNA solution.

21. Transfer DNA solution to a clean 1.5 ml microcentrifuge tube for long-term storage.

**Example 6**

1. Fragments of chromosomal DNA are partially purified by conventional agarose gel electrophoresis on the basis of size.
2. The region of the gel containing the DNA of interest is excised and placed in a microcentrifuge tube containing 3.0:0.2 Tris EDTA buffer.
3. The tube is placed in a 55° heat block until the gel is melted for about 10 minutes.
4. Invert tube several times to mix thoroughly. Add 700 µl of a chaotropic solution containing 4M guanadine isothiocyanate, 0.05 M TRIZMA Base pH 9.5, 0.3 M sodium acetate, 0.4% TRITON X-100, and 16% 2-propanol. Store at about room

temperature for 10 minutes. Since these materials are unlikely to contain RNA contaminants, no precipitation or RNase treatment steps are needed.

5. Precipitate the DNA by adding 500 µl ice-cold 95% ethanol and storing on ice for 10 minutes. Centrifuge the mixture of the chaotropic solution and the DNA/gel solution is to pellet the DNA. This can be done by placing the microcentrifuge tube in a microcentrifuge at maximum speed (typically 12,000-15,000 rpm) for 5-10 minutes at 4°.
6. Remove and discard supernatant. Wash the DNA pellet with 500 µl of 70% ethanol to remove any residual traces of the chaotropic solution and then re-centrifuge at maximum speed.
7. Following centrifugation, remove the supernatant and allow the DNA pellet to dry.
8. The DNA pellet is then dissolved in water or any suitable buffer.